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EXPERIMENTAL STUDIES

Polymeric-Based Perivascular Delivery of a Nitric Oxide Donor Inhibits Intimal Thickening After Balloon Denudation Arterial Injury: Role of Nuclear Factor-kappaB

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OBJECTIVES To examine the effect of a polymeric-based periadventitial delivery of a nitric oxide (NO)-releasing diazeniumdiolate, spermine/NO (SPER/NO), on balloon injury-induced neointimal hyperplasia in rat ileofemoral arteries.

BACKGROUND Reduced local bioavailability and adverse side effects limit systemic administration of NO to modulate vascular response to injury.

METHODS A polylactic-polyglycolic acid polymeric matrix containing 2.5% SPER/NO (w/w) was applied around the injured arteries. Quantitative histomorphometry was performed at day 14, proliferating cell nuclear antigen (PCNA) immunohistochemistry at day 3 to assess effects on smooth muscle proliferation and electrophoretic mobility shift assay to evaluate effects on transcription factor, nuclear factor-kappaB (NF-kappaB).

RESULTS Treatment with SPER/NO reduced the intimal area (0.011 ± 0.009 vs. 0.035 ± 0.006 mm^2 control, $p < 0.01$) and the intima to media ratio (0.089 ± 0.062 vs. 0.330 ± 0.057 control, $p < 0.005$). Spermine/nitric oxide produced a profound inhibition of PCNA-positive cells (>75%, $p < 0.005$) and significantly suppressed the injury-induced activation of NF-kappaB. Vascular cyclic guanosine monophosphate (cGMP) levels were elevated after treatment with the SPER/NO (0.28 ± 0.03 vs. 0.17 ± 0.02 pmol/mg tissue control, $p < 0.01$). The inhibitory effects on neointimal proliferation were localized to the site of application of SPER/NO and were not associated with any changes in platelet aggregation or bleeding time. Neither SPER nor polymer alone had any significant effects on any of the variables examined.

CONCLUSIONS Polymeric-based perivascular delivery of a NO donor produces a marked localized inhibition of neointimal proliferation in balloon-injured arteries. This phenomenon is associated with suppression of NF-kappaB activation and elevation of the vascular cGMP at the site of injury. (J Am Coll Cardiol 2000;35:493–501) © 2000 by the American College of Cardiology

Restenosis, which occurs in 30% to 50% of patients within three to six months, limits the long-term revascularization benefits of coronary angioplasty and other transcatheter interventions (1). Several potent agents that are directed against one or more of the cellular events involved in restenosis have failed to yield any demonstrable clinical benefit (1). Crucial issues that may play a role include the selection of agent(s) and the mode of drug delivery.

Among the several agents that have been proposed to inhibit luminal narrowing following vascular injury, nitric oxide (NO) and its donors may be ideal candidates. Besides its vasorelaxant properties, NO is a potent inhibitor of platelet activation (2), thrombosis (3), vascular smooth muscle cell (VSMC) proliferation (4,5) and migration (6) and extracellular matrix synthesis (7), all key events that contribute to the luminal narrowing following vascular injury. Several recent studies have demonstrated that NO and NO-generating compounds reduce neointimal hyperplasia in experimental models of vascular injury (8–11). There are, however, limitations associated with the systemic administration of NO donors. First, NO is rapidly inactivated by hemoglobin in the circulating blood resulting in limited bioavailability. Second, the adverse systemic hemodynamic and hemostatic effects often preclude administration of biologically effective doses of NO.

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Abbreviations and Acronyms

cGMP	= cyclic guanosine monophosphate
EEL	= external elastic lamina
EMSA	= electrophoretic mobility shift assay
IEL	= internal elastic lamina
NF-kappaB	= nuclear factor-kappaB
NO	= nitric oxide
PCNA	= proliferating cell nuclear antigen
SPER	= spermine
SPER/NO	= spermine/nitric oxide
VSMC	= vascular smooth muscle cell

To overcome these potential limitations, we used a polymeric-based delivery system to examine whether perivascular local treatment with spermine/nitric oxide (SPER/NO), a NO-releasing diazeniumdiolate, inhibits vascular injury-induced neointimal thickening in a balloon ileofemoral artery injury model in rats. The rationale of this approach was based on the enhanced delivery efficiency, prolonged residence time and minimization of systemic side effects with this local drug delivery approach. Recent findings in several animal preparations have corroborated the effective inhibition of luminal narrowing following vascular injury with polymer-based perivascular delivery of antiproliferative agents (11–14). A secondary goal of this study was to determine whether the inhibitory effects of NO on the vascular proliferative response to injury may involve the pleiotropic transcription factor, nuclear factor-kappaB (NF-kappaB), an important modulator of genes involved in the immune and proinflammatory response.

METHODS

Experimental design. All animal experiments were performed according to the animal welfare policy of the American Heart Association and after obtaining approval from the institutional animal care committee. The experimental design is illustrated in Figure 1.

Arterial injury model. Adult male Sprague-Dawley rats weighing 400 to 500 g were anesthetized with pentobarbital inhalation, and the ileofemoral arteries on both sides were surgically exposed up to the first major branch of the femoral artery. A 2F Fogarty balloon catheter (Baxter, Deerfield, Illinois) was introduced from the left carotid artery into the ileofemoral arteries. The balloon was inflated with saline and gently withdrawn to the level of the bifurcation of the aorta. The balloon was deflated and the balloon-induced injury repeated for a total of three times (15). After withdrawal of the catheter, the surgical incision was closed, and the rats were allowed to recover from anesthesia.

Perivascular delivery of NO donor. A local perivascular delivery system composed of a biodegradable biocompatible

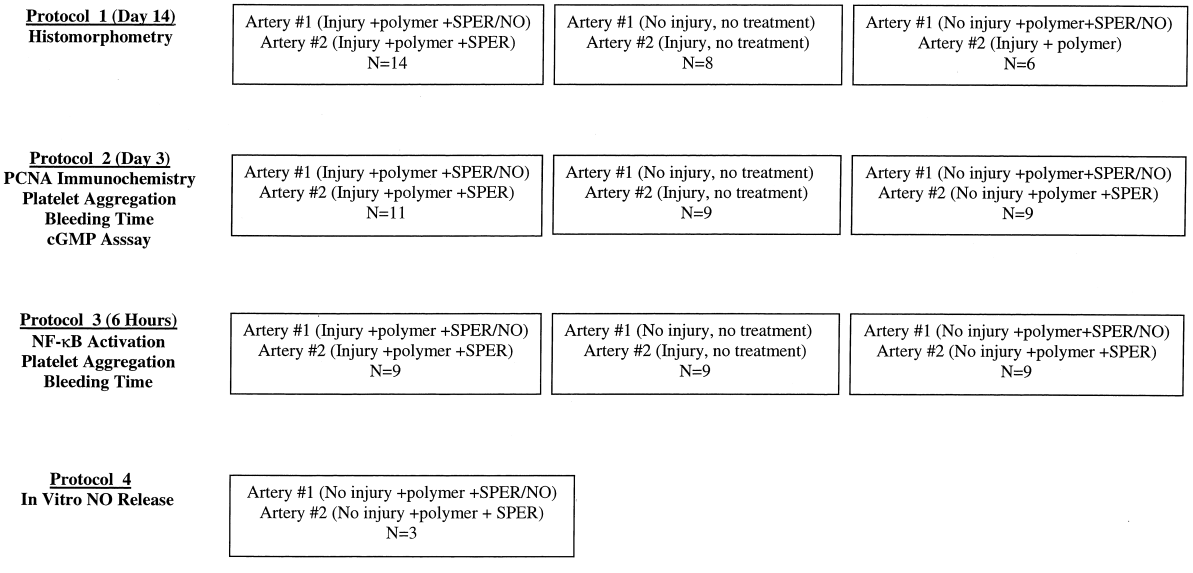

Figure 1. Figure illustrating the experimental design. The study consisted of four separate protocols. In study protocol 1, the effect of NO donor on neointimal thickening was evaluated by histomorphometry at day 14 after balloon injury (n = 28). In study protocol 2, PCNA immunohistochemistry was used to assess DNA synthesis and VSMC proliferation at day 3 after balloon injury (n = 18). Vascular cGMP levels were also measured by radioimmunoassay in protocol 2 (n = 11). In study protocol 3, activation of NF-kappaB, was examined by EMSA and supershift assays at 6 hours after balloon injury (n = 27). Platelet aggregation and bleeding time were also evaluated in some rats in protocols 2 (n = 6) and 3 (n = 5). In study protocol 4, the in vitro release profile of NO form the polymeric delivery system was examined in isolated arteries (n = 3). cGMP = cyclic guanosine monophosphate; EMSA = electrophoretic mobility shift assay; NF-kappaB = nuclear factor kappa B; PCNA = proliferating cell nuclear antigen; SPER/NO = spermine/NO (NO donor); SPER = spermine (vehicle for the NO donor); VSMC = vascular smooth muscle cells.

polymeric material, atrigel, (Atrix Laboratories, Ft. Collins, Colorado), a copolymer of polylactic and polyglycolic acid, was used for the delivery of NO. The gel compound used in the study has a unique reverse-phase gelation property. It exists as a free-flowing liquid below body temperature and solidifies into a viscous mass upon contact with perivascular tissue fluid at body temperature (16). Thus, the polymer gel enables a depot drug delivery in which the NO donor is released over several days as it biodegrades, with complete resorption in about 14 days (16). The NO-releasing diazeniumdiolate used in this study, SPER/NO, releases free NO in a pH-dependent fashion with rapid release at acid pH. Because the copolymer generates small amounts of acid (D-lactic/L-lactic and glycolic acid) by hydrolytic cleavage of their ester linkages during its degradation, magnesium hydroxide (2.5% w/w) was added to the mixture to neutralize the acid pH, thereby enabling controlled release of NO.

Immediately following arterial injury, a mixture of 2.5% w/w of SPER/NO (5 mg), 2.5% w/w of magnesium hydroxide (5 mg) and 200 mg of copolymer (0.2 ml) was applied around the distal half of the injured arteries on one side (injury + polymer + SPER/NO). A mixture of spermine (SPER), the carrier vehicle for the NO donor, and the copolymer was applied to the contralateral injured artery (injury + polymer + SPER) in the first group of rats. In the second group, the artery on one side was injured without any treatment (injury, no treatment), while the other artery was left uninjured without any treatment (no injury, no treatment). The third group had the polymer alone applied around the injured artery on one side (injury + polymer) while the contralateral uninjured artery was treated with the NO donor/polymer mixture (no injury + polymer + SPER/NO).

Histomorphometry. At 14 days after balloon injury, the rats were killed by an intraperitoneal injection of sodium pentobarbital, and the arteries were perfusion-fixed with 1% glutaraldehyde, harvested and then immersion-fixed in 3% glutaraldehyde. The tissue was processed routinely through graded alcohols, cleared in xylene, paraffin-embedded, sectioned (6 μm) and subsequently processed for light microscopy (hematoxylin/eosin). Cross-sectional area within the external elastic lamina (EEL), the internal elastic lamina (IEL) and the luminal area were determined by computerized morphometry using an image analysis software (Optimas 5.1, Bothell, Washington), and the intimal area (IEL-luminal area) and medial area (EEL-IEL) were calculated. Data were expressed as an average of three sections per each arterial segment. Two independent investigators blinded to the experimental group performed morphometric analysis.

PCNA immunohistochemistry. Proliferating cell nuclear antigen (PCNA), a marker of proliferation, was measured by immunocytochemistry in prepared sections for determination of VSMC proliferation that peaks at three days after injury in this model. A commercially available PCNA detection kit (Dako, Carpenteria, California) using a mouse monoclonal antibody against PCNA was used with appropriate positive and negative controls, as described previously (15). Data were presented as PCNA-positive cells/section averaged over three sections per segment.

Electrophoretic mobility shift assay (EMSA). The harvested arteries were collected in ice-cold 0.9% saline and then stripped of adventitia and frozen in liquid nitrogen until nuclear extraction. For optimum extraction and analysis of NF-kappaB, 400 to 500 mg of tissue (derived from approximately six arterial segments) was required. Thus, arterial segments obtained from one limb of each six rats (both arteries in the case of three rats that did not undergo injury) were pooled. A total of 27 rats were studied for NF-kappaB analysis by electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared and their protein concentrations were determined as described previously (15). We assessed NF-kappaB binding at 6 h after injury because we had previously shown it to peak at 6 h in this model of arterial injury with decreased binding observed at 1 and 3 days (15).

For EMSA, nuclear extracts (6 μg) were incubated with [^{32}P]-labeled NF-kappaB oligonucleotide (100,000 cpm, Promega, Madison, Wisconsin) in a buffer containing 0.25 mg/ml of poly (dI.dC) (Roche Molecular Biochemicals, Indianapolis, Indiana), 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM DTT, 2.5 mM EDTA and 20% glycerol (total volume of 30 μl) for 30 min at room temperature. Specificity was determined by the addition of 100-fold excess unlabeled oligonucleotide as competitor. For supershift assay, 15 μl of the antibodies against p50 and p65 subunits of NF-kappaB (Santa Cruz Biotechnology, Santa Cruz, California) were used. Reaction was stopped by the addition of the loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromphenol blue, 0.2% xylene cyanol, 40% glycerol). The DNA-protein complexes were separated on a 4% polyacrylamide gel in a low strength buffer (22.3 mM Tris, 22.3 mM borate, 0.5 mM EDTA) at 4°C. Gels were visualized by autoradiography. Quantitative results of the assays were obtained by densitometry of autoradiography.

Vessel cGMP assay. Arterial segments were harvested at day 3, stripped of adventitia and the drug/copolymer mixture, incubated with 10 μM 3-isobutyl-1-methylxanthine for 1 min, and then immersed in 1 ml of ice-cold 10% trichloroacetic acid followed by sonication (Heat Systems Ultrasonics, Inc., Plainview, New York). Samples were then centrifuged at 10,000 g for 15 min, and the aqueous phase was frozen in liquid nitrogen and stored at −70°C before assay. Each sample was thawed, trichloroacetic acid in the supernatant was extracted by four washes with water-saturated ether, and the sample was evaporated to dryness in a Speed-vac. The sample was reconstituted in buffer and assayed using commercially available radioimmunoassay kits (Amersham, Arlington Heights, Illinois). Protein concentration was determined by Coomassie Plus Assay (Pierce,

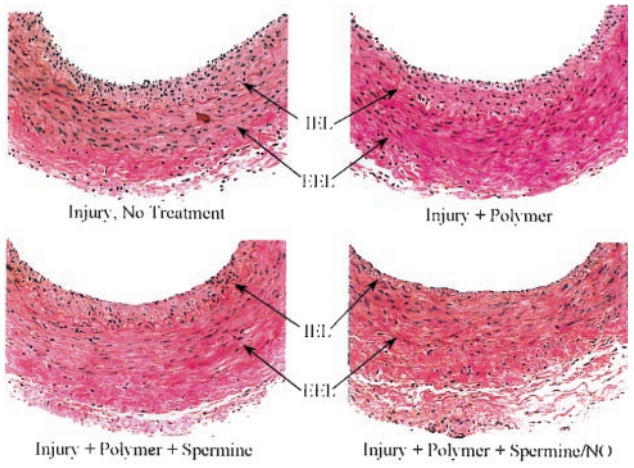

Figure 2. Photomicrographs demonstrating the effects on balloon injury-induced neointimal thickening. EEL = external elastic lamina; IEL = internal elastic lamina; NO = nitric oxide; spermine/NO = NO donor; spermine = vehicle for the NO donor. (Hematoxylin & eosin; × 100, reduced by 70%.)

Rockford, Illinois). Results were expressed as pmol of cyclic guanosine monophosphate (cGMP)/mg protein.

Platelet aggregation and bleeding time. Platelet aggregation was assessed in whole blood by impedance aggregometry pre-and 6 h (NF-kappaB group, n = 5) and 3 days (PCNA group, n = 6) posttreatment with the NO donor (3). Blood (0.5 ml) was collected in tubes containing sodium citrate (0.3%), and aggregation was induced by 5 μg/ml of collagen and quantified as rate of increase in impedance (ohms/min) and maximum increase in impedance ($ohms_{max}$). Bleeding time was performed in anesthetized rats by the tail transection method in five control rats and in treated rats at 6 h (NF-kappaB group, n = 5) and 3 days (PCNA group, n = 6) posttreatment with the NO donor. Bleeding was induced by transection of the tail 5 mm from the tip. The tails were gently blotted with the filter paper, and the time in seconds to cessation of bleeding was measured (17).

Release kinetics of NO. The NO release profile from the polymeric-based perivascular delivery system was estimated in perfused arterial segments in vitro using the acid-reflux chemiluminescence protocol (10). Briefly, a 2 cm-long segment of ileofemoral artery was harvested from a rat, mounted in an organ chamber without removing the adventitia and perfused intraluminally and extraluminally with phosphate buffer maintained at pH 7.4 and 37°C. A mixture of 2.5% w/w of SPER/NO (5 mg), 2.5% w/w of magnesium hydroxide (5 mg) and 200 mg of copolymer (0.2 ml) was applied around the artery. A Whatman #50 (Whatman Inc., Clifton, New Jersey) filter paper was placed around the gel-coated artery to wrap the polymer around the artery. The polymer gelled immediately upon contact with the artery. The perfusate from the artery was collected at baseline and at 15-min intervals for the first 3 h and then every 4 h until 12 h. Nitric oxide and its oxidation product NO_2^- (NO_x) were measured by chemiluminescence detector (model 2107, Dasibi, Glendale, California) after sample reduction in boiling acidic vanadium chloride and reaction with ozone at 98°C. Signals from the detector were analyzed by a computerized integrator and recorded as areas under the curve. Standard curves for the NO_2^- were linear over

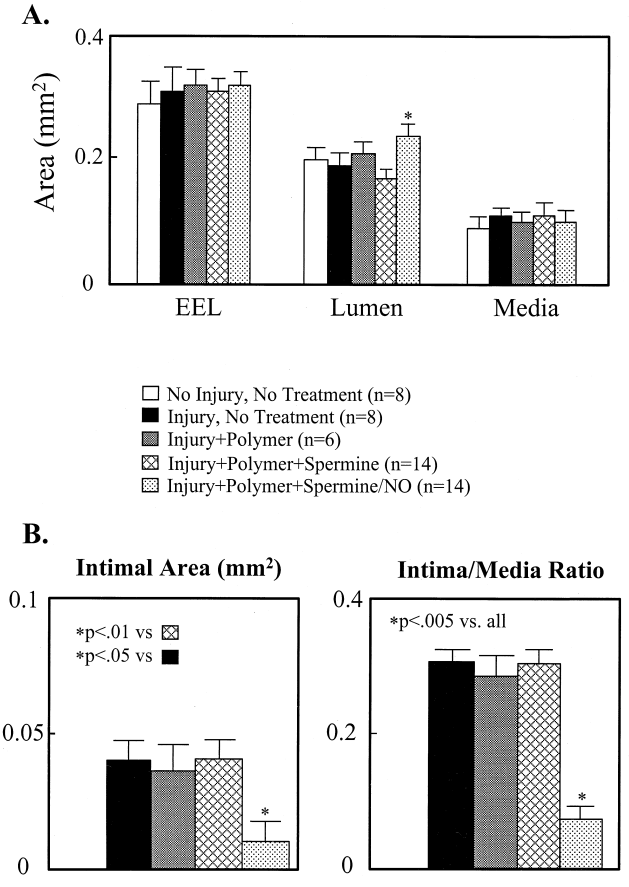

Figure 3 (A and B). Bar graphs demonstrating the effects on histomorphometric parameters. In the first group of rats, the injured arteries on one side were treated with the NO donor-polymer mixture (injury + polymer + spermine/NO) and a mixture of spermine, the carrier vehicle for the NO donor, and the polymer was applied to the contralateral injured artery (injury + polymer + spermine). In the second group, the artery on one side was injured without any treatment (injury, no treatment) while the other artery was left uninjured without any treatment (no injury, no treatment). The third group had the polymer alone applied around the injured artery on one side (injury + polymer) while the contralateral uninjured artery was treated with the NO donor/polymer mixture (no injury + polymer + spermine/NO). Data are mean ± SEM: n = number of animals. P values are based on analysis of variance with Tukey's multiple-comparison test except for **A** where: * is $p < 0.05$ vs. injury + polymer + spermine based on planned single-pair comparison. NO = nitric oxide. Open box = no injury, no treatment (n = 8); solid box = injury, no treatment (n = 8); gray box = injury plus polymer (n = 6); slanted line box = injury plus polymer plus spermine (n = 14); dotted box = injury plus polymer plus spermine/NO (n = 14).

Table 1. Effect of Spermine/NO on Histomorphometric Parameters

	Proximal Site (Segments A, B)		Treatment Site (Segments C, D)	
Area (mm^2)	Control	Treatment	Control	Treatment
EEL	0.428 ± 0.021	0.387 ± 0.022	0.312 ± 0.019	0.322 ± 0.025
Lumen	0.223 ± 0.020	0.198 ± 0.012	0.166 ± 0.019	0.213 ± 0.009*
Media	0.161 ± 0.021	0.154 ± 0.019	0.108 ± 0.005	0.096 ± 0.019
Intima	0.039 ± 0.009	0.043 ± 0.013	0.035 ± 0.006	0.011 ± 0.009**
Intima: Media	0.242 ± 0.016	0.274 ± 0.011	0.330 ± 0.057	0.089 ± 0.062**

Values are mean ± SEM in 14 rats. The arteries were injured by balloon denudation, harvested at day 14 and serially cut into 4 segments (A–D) from the bifurcation of the aorta to immediately proximal to the first major branch of the femoral artery. Morphometric analysis of sections proximal to the site of application of the NO donor (segments A and B) are compared with those at the site of application of the NO donor (segments C and D).

Control group = spermine + polymer; treatment group = spermine/NO + polymer. EEL = external elastic lamina; NO = nitric oxide.

*$p < 0.05$; **$p < 0.005$ vs. control.

the range of 1 nmol to 1 μmol. All sample concentrations of NO_x fell within this range.

Statistical analysis. Data are presented as mean ± SEM. The statistical difference between means was determined by single-factor analysis of variance. A planned single-pair comparison comparing SPER/copolymer versus SPER/NO in injured vessels was performed initially. In addition, Tukey's multiple-comparison test was also performed to examine differences across the various experimental groups (GraphPad Prism 2.1). Probability values of $p < 0.05$ were considered significant. The p values presented are based on Tukey's multiple-comparison test unless specified otherwise.

RESULTS

A total of 94 rats were studied. There were four postprocedural deaths but no bleeding complications. No significant inflammatory response or abscess formation was observed with the use of the copolymer. The biodegradable gel was completely absorbed by 14 days in most animals in protocol 1.

Histomorphometry. Illustrative photomicrographs of histologic cross sections of ileofemoral arteries at the site of application of the NO donor (distal injured segments) are shown in Figure 2, and the results from the quantitative morphometric analysis are shown in Figure 3. Balloon injury of vessels resulted in the development of significant neointimal thickening, which was not affected by treatment with either copolymer alone or the SPER/copolymer mixture. Quantitative analysis revealed that SPER/NO markedly reduced the intimal area ($p < 0.05$ to $p < 0.01$) and the intima to media (I/M) ratio ($p < 0.005$) by about 75%, whereas the medial areas and the areas within the EEL did not differ between the groups. The significant lumen preservation associated with SPER/NO- compared with SPER/copolymer-treated vessels ($p < 0.05$ for planned single-pair comparisons) was primarily due to the reduction in the intimal area since the vessel size (area within the EEL) did not change (Fig. 3A).

Morphometric analysis of segments proximal to the site of application of the NO donor (segment A and B) revealed no significant effects of the NO donor on the intimal area or the I/M ratio (Table 1). Thus, these data show that the marked suppression of neointimal proliferation by the NO donor was localized to the site of application of the drug.

PCNA immunohistochemistry. The quantitative analysis shown in Figure 4 revealed that vascular injury resulted in a significant increase in PCNA-positive cells, compared with the uninjured control arteries. Treatment with SPER/NO, but not SPER alone, produced pronounced inhibition (>75%, $p < 0.005$) in PCNA-positive cells in both injured as well as uninjured arteries.

NF-kappaB activation. As shown in Figure 5, baseline nuclear binding of NF-kappaB was present in the EMSA of nuclear extracts from control, uninjured arteries. This is consistent with the constitutive expression of NF-kappaB in VSMC in vitro (18). The NF-kappaB binding was confined to the media since the injured arteries were completely devoid of endothelium (Evans Blue stain, data not shown)

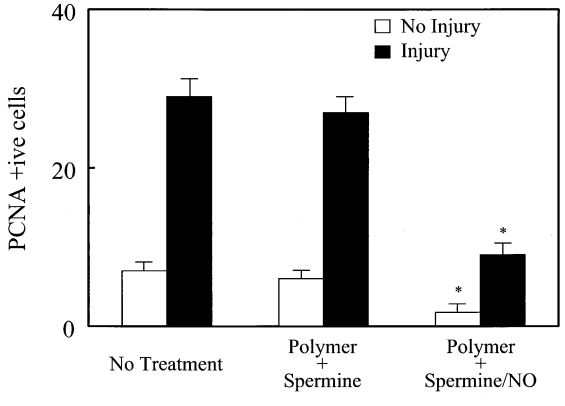

Figure 4. Bar graphs demonstrating the effects on cell proliferation as assessed by PCNA immunohistochemistry. Data (mean ± SEM) are expressed as the number of PCNA-positive cells per section averaged over three sections per artery. N = 6 in each group. *$p < 0.005$ vs. no treatment or polymer + spermine. PCNA = proliferating cell nuclear antigen. Open box = no injury; solid box = injury.

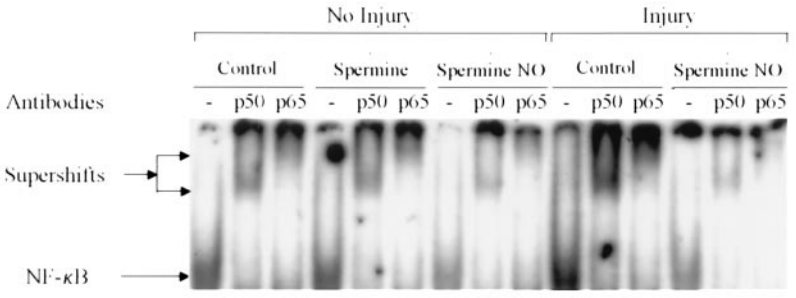

Figure 5. Electrophoretic mobility shift assays demonstrating the effects of NO donor, spermine/NO, on NF-kappaB DNA binding activity (representative of three similar experiments). Supershifted immunoreactive band using antibodies against the p50 and p65 subunits of NF-kappaB are indicated by the **arrows.** NF-kappaB = nuclear factor kappa B; NO = nitric oxide; Spermine = the carrier vehicle for the NO donor.

and the adventitia was completely stripped off. The NF-kappaB nuclear binding in the EMSA of nuclear extracts of uninjured arteries was significantly inhibited by SPER/NO by 38 ± 9% compared with control ($p < 0.01$), but not by SPER (14 ± 7%). Balloon injury produced a marked increase in nuclear binding of NF-kappaB (157 ± 36% compared with no injury), which was markedly inhibited by SPER/NO (61 ± 14%, $p < 0.005$).

The incubation of nuclear extracts with p50 and p65 antibodies reduced nuclear binding and was also associated with mobility retardation (Fig. 5), thus demonstrating the specificity of bands detected in the EMSA.

Vessel cGMP. Vascular cGMP levels were significantly elevated after treatment with the SPER/NO compared with SPER alone in both injured and uninjured arteries (Fig. 6), demonstrating a direct effect of the NO donor on VSMC (since adventitia was removed).

Platelet aggregation and bleeding time. The effect of NO donor on platelet aggregation is shown in Table 2. Spermine/nitric oxide had no significant inhibitory effects on platelet aggregation. The bleeding time did not differ significantly between control rats (211 ± 23 seconds) and the treated rats at either 6 h (234 ± 34 s) or 3 days (213 ± 20 s) following treatment with the NO donor.

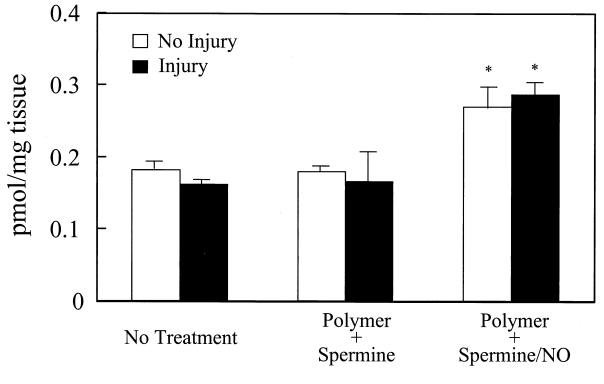

Figure 6. Bar graphs demonstrating the effects on vascular cGMP levels in rats with no treatment (n = 3), rats treated with polymer + spermine/NO (n = 5) and polymer + spermine (n = 3). Data are mean ± SEM. *$p < 0.01$ vs. no treatment or polymer + spermine. cGMP = cyclic guanosine monophosphate. Open box = no injury; solid box = injury.

In vitro NO release. Covering the artery with spermine/NO-copolymer mixture elicited very little NO signal, but this increased markedly with the addition of buffer outside the filter paper cylinder. This indicates that elution of NO from the gel is critically dependent on the aqueous environment maintained at pH 7.4 and 37°C. Graphical integration of the data indicated that the NO release peaked at 40 min (4.5 ± 0.5 μmol) with approximately 95% of total NO release occurring in the first 150 min, and minute quantities still being detectable at 12 h. This is in agreement with the NO release half-life from SPER/NO of about 40 min (19). There was no detectable signal in the perfusate from arteries covered with SPER-copolymer mixture.

DISCUSSION

In this study, we have demonstrated that polymeric-based perivascular delivery of an NO donor produces a marked inhibition of neointimal proliferation in balloon-injured arteries. This phenomenon is associated with suppression of NF-kappaB activation and elevation of the vascular cGMP at the site of injury. Moreover, the inhibitory effect of the NO donor on neointimal proliferation is confined to the site of application, thereby demonstrating a local effect of NO delivery. Although inhibition of neointimal proliferation by NO or NO donors has been observed previously (8–10,20), the findings in this study are novel in two respects. First, this is the first report of inhibiting neointimal proliferation using the novel local polymeric-based perivascular delivery of NO. Second, the inhibitory effect of NO on balloon injury–induced NF-kappaB activation has not been reported previously in vivo.

NO donor. Spermine/nitric oxide is one of several new NO-releasing diazeniumdiolates (compounds containing the [N (O) NO]-functional group, formerly called 'NONOates') (19) that exhibit potent vasorelaxant (21),

Table 2. Platelet Aggregation Responses

	Ohms/Min		$Ohms_{max}$	
	Pre-Rx	Post-Rx	Pre-Rx	Post-Rx
6 h (n = 5)	4.0 ± 0.3	3.4 ± 0.5	11.8 ± 1.1	10.5 ± 2.2
3 days (n = 6)	4.5 ± 0.5	3.9 ± 0.4	13.3 ± 0.9	12.1 ± 1.6

Values are mean ± SEM. Platelet aggregation responses were measured in citrated arterial blood pre- and 6 h and 3 days posttreatment with spermine/NO as described in the Methods section. All P = ns (Student paired *t* test).

antiplatelet (3) and antimitogenic effects (5) in vitro and in animal models. The NO-releasing diazeniumdiolates release free NO in a pH-dependent fashion with predictable first-order kinetics. Their biological half-lives range from 2 s to several days (19); they exhibit greater potency (19), and, thus, they may offer a better therapeutic potential as antiatherogenic agents compared with the conventional NO donors such as nitrates and nitroprusside.

Effects of NO on NF-kappaB. The transcription factor, NF-kappaB, plays an important role in the transcription of a variety of genes, primarily the ones involved in the immune and proinflammatory response (22,23). Nuclear factor-kappaB has been shown to be involved in the cytokine-induced expression of adhesion molecules and monocyte-colony stimulating factor in endothelial cell cultures (22,23), and, in a rat balloon injury model, we recently demonstrated the involvement of NF-kappaB pathway in VSMC proliferation and neointimal hyperplasia in injured arteries (15). In this study, we have confirmed our previous observation of increased vascular NF-kappaB activity induced by injury. The potential mechanism of this effect may be an increase in the oxidant stress or a cytokine induced by mechanical injury, which may trigger the degradation of IkappaBalpha (22–25) with consequent dissociation, nuclear translocation and DNA binding of p50 and p65 subunits of NF-kappaB (as supported by the EMSA data). The finding that NO inhibits NF-kappaB activity is generally in agreement with observations from previous in vitro studies (24–26). However, the new finding in this study is that NO suppresses NF-kappaB activity in injured vessels in vivo. The exact mechanisms by which NO inhibits NF-kappaB activity in vivo is beyond the scope of this study. However, previous studies in vitro suggest that NO may reduce proteolytic degradation of the stabilizing subunit, IkappaBalpha, either via a direct dephosphorylating effect or indirectly via its antioxidant effect (24–26).

There are several noteworthy features of this study. First, a relatively short (<3-h) arterial-wall exposure to the NO donor (based on the NO release studies in vitro) resulted in a prolonged (2-weeks) biologic effect, with the NO donor-treated arteries demonstrating little neointimal formation. Certain molecular events initiated during this critical time period may be essential for vascular lesion formation. Thus, interventions such as NO that inhibit these acute-phase events (including NF-kappaB activation) may suppress downstream events in the response-to-injury cascade resulting in sustained biologic effects. An alternative explanation is that the measured NO_x activity in vitro is likely underestimating the duration of action of the NO donor/polymer mixture in vivo as evidenced by increased cGMP levels at day 3. Second, the effect of NO delivery was remarkably local. While the entire length of the ileofemoral artery was ballooned during the study, the gel containing NO donor was applied only to the distal part of the artery, and it was only this arterial segment that showed suppression of neointimal proliferation. The lack of effects of the NO donor on neointimal proliferation in the contralateral injured artery treated with the vehicle, SPER, alone is consistent with the local effect of the NO donor and excludes the involvement of a systemic pathway. Third, periadventitial delivery of NO effectively inhibited vascular lesion formation after endoluminal balloon-denudation injury of rat ileofemoral arteries. The intraluminal concentration of NO delivered by this route was in the range (4 μM) that exhibits antiproliferative effects on VSMC in vitro (4,5). The medial concentration of NO would be expected to be higher than the luminal concentration and therefore inhibit medial SMC proliferation more markedly. It is possible that NO acted directly on the medial SMC, and the adventitia was simply a convenient route of administration. However, based on the emerging data regarding the direct role of adventitial cells in neointimal formation, it is likely that inhibition of these cells by periadventitial delivery of NO and other potent antiproliferative agents may offer a more effective therapeutic strategy. Fourth, the inhibitory effect of NO on neointima formation appeared to be equal in magnitude to that observed on DNA synthesis. This indicates that the antiproliferative effects of NO might primarily contribute to its inhibitory effects on neointima formation. However, this study does not address the effect of NO on VSMC migration. We, therefore, cannot completely exclude the role of NO's known antimigratory effects (6) in this study. Fifth, the lack of effects on platelet aggregation and bleeding time excludes the possibility that perivascularly delivered NO suppressed neointimal proliferation by altering systemic platelet and coagulation function. However, since platelet adhesion on the injured arterial surface is important in the proliferative response to injury, it is likely that inhibition of local platelet deposition by the NO donor may have contributed to suppression of neointimal hyperplasia in this study. Sixth, the polymer used in this study was noted to induce a severe inflammatory

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reaction in previous studies in rabbits (27) and swine (28) but not in rats (13). We did not see a significant inflammatory response in our rats treated with the polymer. Likely explanations for the differences include the particle size of the polymer, systemic versus perivascular administration and species variability. The biocompatibility of the polymer is further supported by the lack of incremental neointimal formation.

Study limitations. There are several limitation of this study. Although the rat artery balloon injury model is not an optimal model of human disease, and extravascular delivery of drugs is not yet generally feasible, this study provides "proof of concept" evidence that perivascularly delivered NO may be important in inhibiting neointimal hyperplasia. The precise role of NO and its modulation of NF-kappaB activation on the vascular proliferative response to injury is not clear. These studies are extremely difficult to accomplish in vivo. However, we and others have recently demonstrated in studies in vitro that the inhibitory effects of NO on VSMC proliferation (25,29) and endothelial cell activation (24) involve the NF-kappaB pathway. The release kinetic profile for NO from perivascularly administered NO donor-copolymer mixture was evaluated in an in vitro model. Drugs can be cleared from the perivascular space by 1) transmural diffusion, 2) absorption by extraarterial microvessels, and 3) absorption by lymphatics (30). The in vitro model obviously precludes identification of such mechanisms by which NO is cleared from the perivascular space and enters vascular tissue.

Implications. Recent studies in experimental models and in humans indicate that NO and NO-generating compounds possess potent vasorelaxant (2), antiplatelet (2,3) and antiproliferative properties (4,5,8–11). These observations have important implications for these compounds as effective therapeutic agents for treatment of vasculoproliferative states such as restenosis after coronary interventions. However, rapid inactivation by hemoglobin in the circulating blood, thereby limiting high target concentration of NO for optimal biologic effects, and adverse hemodynamic effects preclude systemic administration of NO donors. Furthermore, low efficiency and rapid redistribution of the infused material have limited local endovascular delivery of therapeutic agents to modulate the vasculoproliferative response to injury. In this study a local perivascular delivery was carried out by placing an NO donor–containing polymer in close proximity with the vessel wall, ensuring a high local concentration of NO and minimizing blood flow washout and rapid inactivation of NO. Such a delivery system may also offer the advantage of dissociating the desired local antiplatelet and antiproliferative effects from undesired adverse systemic hemodynamic and hemostatic effects. We are currently adopting a similar perivascular strategy in a porcine model using intrapericardial delivery of therapeutic agents via a percutaneous subxyphoid approach to modulate the coronary arterial response to injury.

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